

Immunoassays for the
Diagnosis of Rotavirus and
Norwalk Virus Infections

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IMMUNOASSAYS FOR THE DIAGNOSIS OF ROTAVIRUS AND NORWALK VIRUS INFECTIONS

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16. ABSTRACT Acute viral gastroenteritis is an extremely common illness that affects all age groups throughout the world and occurs in both epidemic and endemic forms. Among illnesses affecting United States families, it is second in frequency only the common cold. The disease is usually self-limited, although it can be lethal to the malnourished, elderly, debilitated or infant patient. The two viral pathogens currently known to have medically important etiologic roles in human gastroenteritis, rotavirus and Norwalk virus, were discovered during the 1970's by electron microscopy. Development of rapid diagnostic immunoassays for these viruses has led to an understanding of their epidemiology and medical importance. This book chapter discusses the radioimmunoassay (RIA) and the enzyme immunoassay (EIA) procedures used to detect these viral agents.		
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Acute viral gastroenteritis (reviewed in Blacklow and Cukor, 1981) is an extremely common illness that affects all age groups throughout the world and occurs in both epidemic and endemic forms. Among illnesses affecting United States families, it is second in frequency only to the common cold. The disease is usually self-limited, although it can be lethal to the malnourished, elderly, debilitated or infant patient. The two viral pathogens currently known to have medically important etiologic roles in human gastroenteritis, rotavirus, and Norwalk virus, were discovered during the 1970's by electron microscopy (Bishop et. al., 1973; Kapikian, et. al., 1972). Development of rapid diagnostic immunoassays for these viruses has led to an understanding of their epidemiology and medical importance.

I. ROTAVIRUS

A. Epidemiology and Virology

Rotavirus infection is the single most important cause of acute diarrheal illness requiring hospitalization of infants and young children, and accounts for 40-60% of such hospitalization in temperate climates. The virus also appears to have a major role in gastroenteritis in infants and young children not requiring hospitalization. Infection occurs world wide usually in sporadic but occasionally in epidemic forms. In temperate climates, the peak incidence of rotaviral disease occurs during the cooler months whereas in tropical areas cases occur year round. Asymptomatic rotavirus infections occur frequently in adult contacts of ill children. Symptomatic adult rotavirus infections have been reported among parents of ill children and also among adults who lack contact with children and in travelers. Transmission of rotavirus is from person to person by the fecal oral route. The incubation period is 1 to 3 days. The virus is detectable in feces during acute illness and is commonly shed

for up to 8 days after the onset of symptoms and occasionally longer. Nosocomial spread of infection among hospitalized pediatric patients and medical personnel is well documented. Prevalence studies indicate rapid acquisition of serum antibody with most individuals possessing anti-rotavirus antibody by 3 years of age.

Serologic assays have been useful for epidemiologic studies, but because of the virtually universal presence of preexisting serum antibody, the diagnosis of an episode of illness depends upon the detection of rotavirus in diarrheal stool specimens. A rapid diagnosis of rotavirus illness provides the physician with useful information for formulating a prognosis, may prevent unnecessary use of antibiotic therapy and prolonged hospitalization, and allows for the institution of appropriate control measures.

Most human rotaviruses are not directly cultivatable in cell culture. Recently a human rotavirus strain has been adapted to replication in cell culture after 11 passages in gnotobiotic pigs (Wyatt et al, 1980). Other strains have been induced to replicate in vitro after treatment with trypsin, concentration by ultracentrifugation and approximately six blind passages in cell culture (Urusawa, et. al., 1981; Sato et. al., 1981). Even with the use of these special and time consuming techniques, approximately 50% of the isolates currently remain non-cultivable. Therefore, at the present time tissue culture isolation of human rotavirus remains an exciting research tool but not a practical diagnostic technique.

The characteristic morphology of rotaviruses is readily recognized in negatively stained stool preparations under the electron microscope. EM studies resulted in the original identification of rotavirus (Bishop et. al., 1973). This technique has been used extensively for rotavirus diagnosis and remains the standard by which other assays must be judged. However, the usefulness of EM is limited by the requirement for highly specialized equipment and personnel as well as by the difficulty in handling large numbers of specimens.

The diagnostic test of choice for most situations is the solid phase immunoassay. It offers the possibility of same day or next day diagnosis of rotavirus infection and can be performed on a large scale with routinely available equipment and personnel. In this assay the stool specimen to be tested is exposed to a solid phase which has been coated with antirotavirus antibody. After an absorption period and washing, a labeled virus-specific detection antibody is used to demonstrate the presence of rotavirus bound to the solid phase.

B. Considerations in Establishing Rotavirus Solid Phase Immunoassays

1. Choice of serum reagents

At least 3 serotypes of human rotavirus have been identified based on virus neutralization assays (Wyatt et. al., 1982). In addition, two different antigenic specificities, designated as subgroups, have been identified by differential reactivity of human rotaviruses in immunoassays (Kapikian et. al, 1981). All human rotaviruses seem to exhibit the same clinical and epidemiological patterns and the grouping or typing of isolates would serve no practical diagnostic purpose at this time although it is useful for following epidemiologic trends of infection.

Several approaches have been utilized for the production of high titered antirotavirus serum for use in immunoassay techniques. Convalescent human sera have not been found to be of sufficiently high titer for practical use and therefore hyperimmune animal sera have been used. In one approach human rotavirus is purified directly from human diarrheal stool specimens and is used as antigen for the immunization of laboratory animals (Kjeldsberg and Mortensson-Egnurd, 1982; Middleton et.al.,1977; Grauballe et al., 1981, Obert et.al., 1981; Sarkkinen et.al.,1979). The major drawback of this approach is that the hyperimmune serum produced may contain antibody reacting with human stool components which copurified with the virus. This would result in higher background reactions and perhaps false positive reactions with certain stools which contain high concentrations of the reacting antigen. To circumvent this problem, a second approach has been taken in which human rotavirus is used to induce illness in gno-

tobiotic calves. The virus is then purified from calf stool, thus avoiding contamination with human stool components and permitting its use as a cleaner immunogen (Kalica et.al.,1977; Yolken et.al.,1977a). The third approach exploits the presence of a group specific antigen shared by mammalian rotaviruses. Several of the animal rotaviruses such as the simian SA-11 and bovine NCDV are readily propagated in tissue culture and can be purified as antigen for the production of hyperimmune sera. (Cukor et.al.,1978;Rubinstein and Miller, 1982;Hammond et.al.,1982). Since gnotobiotic animals are not readily available, the third approach is currently preferred for most laboratories.

In order to avoid further problems associated with non-specific reactivity of serum reagents, it is preferable that the coating and detection antibodies for immunoassays be prepared in different animal species. In this way, undesired reactivity present in only one of the reagent sera would not interfere with the test. Goats, rabbits, and guinea pigs have all been used successfully for anti-rotavirus serum production. Not all individuals of a given species produce satisfactory hyperimmune serum. Therefore, several animals should be immunized and their sera separately tested in the immunoassay. Other assays such as complement fixation are not adequate in our experience for the screening of sera for use in solid phase immunoassays. (Table 1).

2. Choice of solid phase

Flexible 96 well polyvinyl chloride microtiter plates have been used with success when goat serum is employed as a coating antibody. Hard polystyrene plates may also be used with other animal sera. Individual polystyrene beads offer an advantage when small or variable numbers of specimens are to be tested, but they are slightly more laborious to handle. The flexible plates may also be cut up to obtain the desired number of wells for each test.

3. Sample preparation

Only diarrheal stools from the acute phase of illness are likely to contain virus. Stool samples may be stored at -70°C. When stools are proces-

sed for study, no serum should be used in the diluent unless it has been shown to be negative for rotavirus antibody. In our laboratory, an approximate 2 to 10% suspension of stool is prepared in veal infusion broth supplemented with 0.5% bovine serum albumin, mixed on a Vortex apparatus for 1 min. and clarified by centrifugation at 200xg for 15 min. (Blacklow and Cukor, 1980). Rectal swabs have been used with somewhat less success than stool specimens (Brandt et.al., 1981). The incorporation of EDTA into the suspension medium (0.025 M) has been reported to increase the sensitivity of the test for some specimens, presumably by exposure of additional latent group specific antigen on the surface of the virus particles in the presence of the chelating agent (Hammond et.al., 1982; Beards and Bryden, 1981).

4. Choice of test conditions

A wide variety of test conditions have been used in different laboratories. Incubations have usually been performed at either room temperature or 37°C for periods ranging from 1h to 12h. Recent studies indicate that incubation times of only 5 to 10 minutes may be sufficient (Yolken and Leister, 1982) in that it appears that when high titered reagents are used at optimal concentrations (as predetermined by checkerboard titration) the incubation conditions are not critical and will largely depend on the convenience of the operator.

E. Choice of an indicator system

Although the use of radio-iodinated detection antibody is convenient in a research situation where a planned number of specimens are to be tested and iodination facilities are available, radioimmunoassay (RIA) is less practical in a clinical diagnostic setting. The reason for this is the short shelf life of radioactive antibody and the need for special facilities for handling and disposing of radioactive materials. The enzyme immunoassay (EIA) uses a stable enzyme-antibody conjugate as a detection system. The addition of a

chromogenic substrate allows for the convenient quantitation of bound antibody with the use of a spectrophotometer. The availability of specially modified spectrophotometers (EIA readers) makes the EIA system the most practical for clinical laboratories.

The most commonly used enzymes are alkaline phosphatase and horseradish peroxidase. Alkaline phosphatase has the disadvantage of being expensive; also some clinical material may contain endogenous enzyme activity which could contribute to higher background values. Peroxidase is cheap and readily available; however, the enzyme is inhibited by the presence of metal ions, bacterial contamination, and antibacterial agents such as methanol and sodium azide. Betagalactosidase has also been used in some EIA systems. It offers the advantage of being absent from clinical specimens, but because of the low specific activity of available preparations color development is much slower than for the other enzymes. The various enzymes and substrates available for EIA systems have recently been reviewed (Yolken, 1982).

F. Choice of Direct or Indirect Assay

Thus far our discussion has been confined to the direct immunoassay for rotavirus. In its indirect modification (Yolken et.al., 1977b), the antibody coated solid phase is reacted with the test specimen as in the direct test. Next an unlabeled antirotavirus serum (derived from a species other than the source of the coating antibody) is added. After incubation and washing steps, a labeled antiglobulin directed against the animal species of the second antibody is utilized as the detection antibody. With the inclusion of appropriate controls (see below) the indirect assay system has been used successfully for the detection of rotavirus (Sarkkinen et.al., 1979, Brandt et.al., 1981; Yolken et.al., 1980). By analogy with fluorescent antibody tests, the indirect EIA includes an attenuation step and therefore provides the theoretical

advantage of increased sensitivity. In addition, it precludes the necessity of labeling each detection antibody preparation by allowing the use of a single labeled antiglobulin for many different types of immunoassays.

When direct and indirect EIA tests for rotavirus using the same reagents were compared (Hammond et.al.,1982), the theoretical advantage of increased sensitivity with the indirect test was not realized. Rotavirus is excreted by ill children in copious amounts (Vesikari et.al., 1981), which have been estimated to approach 1 mg of viral antigen per ml of diarrheal stool. In light of this, increased sensitivity is probably not an important consideration in the routine clinical diagnostic situation. The indirect test is somewhat less convenient than the direct assay because extra incubation and washing steps are required. However, it appears that the major drawback of the indirect test is the repeated observation of an increased incidence of non-specific reactions (see below, Hammond et.al.,1982;Brandt et.al.,1981, Yolken et.al.,1977b) and high background levels of reaction (Sarkkinen et.al.,1979;Hammond et.al.,1982), reportedly resulting in a test of low sensitivity and specificity (Hammond et.al.,1982; Brandt et.al.,1981).

G. Non-Specific reactions and selection of controls

In the original design of the solid phase immunoassay for rotavirus (Kalica et.al.,1977; Yolken et.al.,1988a), the reactivity of test specimens was compared to the reactivity of a panel of known negative stool samples. A confirmatory test was recommended for determining the status of weakly positive specimens. The confirmatory test, consisting of exogenous blocking (neutralization) of sample reactivity with a high titered antirotavirus serum, was laborious and felt to be unnecessary for routine use with each specimen tested. However, after several years of experience with this test, it became apparent that serious problems existed in the frequency of nonspecific reactions (Brandt et.al.,1981;Yolken et.al.,1977b;Yolken and Stopa,1979). Non-specific reactions to goat serum were first recognized in stools from patients living in certain geo-

graphic areas, especially the Far East, and thought to relate to their diet. Other stools contained nonspecific antiglobulin activity related to an IgM molecule present in the specimen. To circumvent these problems, normal goat serum and a reducing agent were added to all stool specimens (Yolken and Stopa, 1979). Others have attempted to eliminate nonspecific reactions by the addition of EDTA or BSA or by neutralization of the pH of the stool specimen (Middleton et.al.,1977, Hammond et.al.,1982). Some but not all nonspecific reactions could be eliminated by these measures. In an extensive study reported in 1981 from the Children's Hospital National Medical Center in Washington,D.C. (Brandt et.al.,1981), it was found that among 5,626 fecal specimens tested, 1,344 gave positive results with the indirect EIA test which could not be confirmed by electron microscopic visualization of rotavirus particles, blocking EIA, confirmatory EIA or a combination of these methods. Thus, 73% of the 1,834 presumptively positive EIA tests were in fact not positive. False-positive EIA reactions were especially common with specimens from hospitalized young infants in a tertiary care nursery. It was postulated that nonspecific binding of rotavirus antibody to intestinal bacteria or their products such as staphylococcal protein A may have been the basis of some of the false positive reactions observed.

Based on this experience it is apparent that measures such as the addition of reducing agent are inadequate since they prevent only some kinds of nonspecific reactions. It is also inadequate to rely on a panel of known negative stools as controls. Furthermore, not only borderline specimens but all potential positives need to be confirmed.As in the system originally employed for the detection of Norwalk virus (Greenberg et.al.,1978), the reaction of each stool specimen in the well coated with rotavirus positive (post-immunization)serum is compared with the reaction of the same specimen in a control well coated with rotavirus negative (pre-immunization) serum (Yolken et al 1980; Kapikian et.al.1979). When such a confirmatory test was included in the indirect EIA at Children's Hos-

pital in Washington (Brandt et.al.,1981), false-positive results were not encountered in 400 consecutive tests.

H. Calculation of cut off value for positive results

A variety of calculations have been used to determine the cut off value for positive specimens. In an RIA system, positive/negative (P/N) values are often calculated by dividing the counts bound to the experimental well (P) by the counts bound to the control well (N). P/N values ≥ 2 are usually considered to be positive. (Kalica et.al.,1977;Cukor, et.al.,1978).

In an EIA system where control absorbance values may be lower and more variable, a P minus N calculation may be used (Yolken et.al.,1977a;Rubinstein and Miller,1982). It is clear that there is not a single method for calculation of results that applies to all of the different immunoassays; rather, for each new assay developed the test and calculation method must be validated by testing large numbers of positive and negative specimens and comparing the results with a standard test, preferably EM (Kjeldsberg and Mortensson-Egnund,1982; Middleton et.al.,1977; Grauballe et.al.,1981;Obert et.al.,1981; Sarkkinen et.al., 1979;Kalica et.al.,1977; Yolken et.al.,1977a; Cukor et.al.,1978; Rubinstein and Miller, 1982; Hammond et.al.,1982; Brandt et.al.,1981; Beards and Bryden,1981; 1981,Wall et.al.,1982).

I. Commercially available tests

As of the middle of 1982, one rotavirus EIA kit is licensed for diagnostic use. (Rotazyme,Abbott Laboratories,North Chicago,IL) and two others are available for investigational use (Dakopatts,Acurate Chemical Corp.,Westbury,NY and Enzygnost,Calbiochem.Corp. LaJolla,CA). Table 2 compares the design of these 3 tests with the current versions of the indirect EIA used at the Johns Hopkins University (Yolken et.al.,1981). and the RIA test used at the University of Massachusetts Medical School (Blacklow and Cukor,1980). Large numbers of specimens have been tested by the Rotazyme assay in several laboratories (Rubinstein and Miller, 1982; Hammond et.al.,1982; Beards and Bryden, 1981;Wall et.al.,1982,Yolken and

Leister, 1981) with favorable reports as to the sensitivity and specificity of the test. The test appears to be somewhat less sensitive than most EM or indirect EIA assays. Despite the manufacturer's lack of providing a confirmatory test, problems of specificity have not been reported for the Rotazyme. Apparently the direct test design and special formulations of sample diluent and wash solutions are sufficient to eliminate most false positive results. Laboratories may elect to use an exogenous confirmatory blocking test with any high titered antirotavirus serum in conjunction with the Rotazyme test. Only limited experience with the other 2 commercial assays has been reported (Kjeldsberg and Mortensson-Egnund, 1982; Grauballe et.al., 1981; Yolken and Leister, 1981). Both tests suffer from the theoretical disadvantage of using serum from the same animal species as both coating and detection antibodies.

III. NORWALK VIRUS

Norwalk virus is responsible for a high proportion of outbreaks of acute gastroenteritis among older children and adults. This virus has been associated with 42% of 74 outbreaks of acute nonbacterial gastroenteritis in the U.S. studied epidemiologically by the Centers for Disease Control (Kaplan et.al., 1982). These outbreaks occur during all seasons and in a variety of locations including families, elementary schools and colleges, nursing homes, cruise ships, swimming pools, recreational camps and restaurants. The virus is usually transmitted by person to person contact or contaminated water and occasionally food.

It would clearly be desirable for State Laboratories and other public health authorities to have available a diagnostic test for Norwalk virus. This is difficult for several reasons: the fact that the virus is shed in relatively small amounts in feces, the inability of the virus as of the present time to replicate in vitro or in laboratory animals, and the failure of current technology to prepare highly purified virus from human stool samples. Because of its small size, the virus can only be visualized by the laborious and technically difficult immune electron microscopic (IEM) procedure (Kapikian et.al., 1972). The lack of purified viral antigen precludes the possibility of preparing hyperimmune animal

serum which would be useful in an immunoassay.

Virtually all that we know about Norwalk virus is based on the one model system available for study of the pathogenesis of the infection, human volunteers. When these individuals orally ingest the virus, they experience an illness clinically indistinguishable from the naturally occurring disease. Stool and serum specimens generated by volunteers have provided the only laboratory reagents currently available to identify the virus and are used for RIA and IEM tests. These human agents also permit the quantitation of antibody responses in sera and intestinal secretions. Unfortunately because the reagents are specimens from human volunteers, the assay for Norwalk virus is available in only a few research laboratories.

The RIA test for Norwalk virus (Greenberg et.al.,1978; Blacklow et.al, 1979) is similar in principle to the confirmatory RIA test described above for rotavirus. In brief, wells of microtiter plates are coated with acute and convalescent phase sera from a selected volunteer who has experienced experimentally induced Norwalk virus illness. The stool samples to be tested are then added, and finally convalescent phase serum from a different volunteer is used as the source of radioiodine-labeled detection antibody. It should be noted that the serologic assay for Norwalk virus is more sensitive than the antigen test and that most Norwalk outbreaks have been primarily identified by the findings of sero-conversions.

Circumventing the use of human derived reagents for Norwalk virus diagnosis and developing RIA tests for other 27nm Norwalk-like viruses which may be important in human disease (Blacklow and Cukor, 1981) are currently two major goals of research in the field of viral gastroenteritis.

TABLE 2
IMMUNOASSAYS FOR ROTAVIRUS

TEST:

FEATURE	ROTAZYME	DAKOPATTS	ENZYGNOST	JOHNS HOPKINS	U. MASS.
OLID PHASE:	polystyrene beads	polystyrene microplates	polystyrene microplates	polyvinyl microplates	polyvinyl microplates
APTURE Ab:	guinea pig anti-SAll (tissue culture derived)	rabbit anti-HRV (human stool de- rived) JK control normal rabbit serum	rabbit anti- SA-11 (tissue culture derived)	goat anti-HRV (gnotobiotic calf stool de- rived) OR control normal goat serum	goat anti-SAll (tissue cultui derived) OR control normal goat serum
12.					
SECOND Ab:				guinea pig anti-HRV	
DETECTION Ab:	rabbit anti-SAll- peroxidase	rabbit anti-HRV- peroxidase	rabbit anti-NCDV- alk.phosph.(tis- sue culture derived)	rabbit anti- guinea pig im- munoglobulin alk.phos.	guinea pig anti-SAll- 125 I
CONFIRMATORY TEST:	non available	included	non available	included	included

Table 1
a)
Selection of Hyperimmune Serum

<u>SERUM</u>	<u>COMPLEMENT FIXATION TITER</u>	<u>RIA For SA-11 (P/N)</u>	<u>ACCURACY OF RIA FOR STOOL</u>
GP A	1:8,192	37.0	100%
GP B	1:16,384	5.6	50%
GP C	1:4,096	10.1	50%
GP D	1:8,192	16.3	50%
GP E	1:8,192	21.5	100%

a)

Sera from 5 hyperimmunized guinea pigs were evaluated in 3 ways i) the titer of CF antibody to SA-11 virus; ii) P/N ratios obtained by reaction in RIA with cell culture grown SA-11 virus; iii) capability to distinguish by RIA between 4 EM positive and 4 EM negative human stool specimens.

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